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Loss of the Ecdysteroid-Inducible E75A Orphan Nuclear Receptor Uncouples Molting from Metamorphosis in *Drosophila*

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Summary

Isoform-specific null mutations were used to define the functions of three orphan members of the nuclear receptor superfamily, E75A, E75B, and E75C, encoded by the *E75* early ecdysteroid-inducible gene. *E75B* mutants are viable and fertile, while *E75C* mutants die as adults. In contrast, *E75A* mutants have a reduced ecdysteroid titer during larval development, resulting in developmental delays, developmental arrests, and molting defects. Remarkably, some *E75A* mutant second instar larvae display a heterochronic phenotype in which they induce genes specific to the third instar and pupariate without undergoing a molt. We propose that ecdysteroid-induced *E75A* expression defines a feed-forward pathway that amplifies or maintains the ecdysteroid titer during larval development, ensuring proper temporal progression through the life cycle.

Introduction

Ecdysteroids function as key temporal signals in *Drosophila*, directing each postembryonic transition in the life cycle (Riddiford, 1993; Thummel, 2001). Ecdysteroid pulses at 1 day intervals during the first and second larval instars trigger molting of the cuticle, accommodating the growth that occurs during these stages. A high-titer ecdysteroid pulse 2 days after the molt to the third instar triggers puparium formation, signaling the onset of prepupal development and metamorphosis. This is followed by another ecdysteroid pulse, approximately 10 hr after puparium formation, which triggers adult head eversion and the prepupal-pupal transition. A neuropeptide signal from the brain to the endocrine organ of the insect, the ring gland, triggers the release of relatively inactive ecdysteroids into the hemolymph that are converted by peripheral tissues into more active forms of the hormone, primarily 20-hydroxyecdysone (20E; Gilbert et al., 1996).

The ecdysteroid signal is transduced by a heterodimer of two members of the nuclear receptor superfamily, EcR and the RXR ortholog, USP (Thomas et al., 1993; Yao et al., 1992, 1993). This hormone/receptor complex activates cascades of gene expression, as first defined by studies of the puffing patterns of the giant larval salivary gland polytene chromosomes (Ashburner et al., 1974). Ecdysteroids directly induce the formation of about a half dozen early puffs. The protein products of these puffs induce more than 100 late puffs scattered throughout the genome. The late puff products, in turn, are thought to act as effectors that direct appropriate biological responses to each ecdysteroid pulse during development (Russell and Ashburner, 1996).

Molecular characterization of three early puff genes has shown that they encode transcription factors, fulfilling a central prediction of the hierarchical model of ecdysteroid action. The *Broad-Complex* (*BR-C*), responsible for the 2B5 early puff, is a complex genetic locus that encodes a family of zinc finger proteins (DiBello et al., 1991). Null mutations that inactivate all three essential *BR-C* subfunctions lead to prolonged third instar larvae that fail to pupariate, while mutations that affect only a single subfunction result in defects in imaginal disc morphogenesis, larval tissue cell death, and lethality during prepupal and pupal stages (Kiss et al., 1988; Restifo and White, 1992). *BR-C* mutations also have widespread effects on early and late ecdysteroid-inducible gene expression, consistent with a central role for this gene in transducing the ecdysteroid signal (Guay and Guild, 1991).

The two best-characterized early puffs, at 74EF and 75B (Ashburner et al., 1974), also encode ecdysteroid-inducible transcription factors. *E74*, from the 74EF puff, consists of two overlapping transcription units, *E74A* and *E74B*, that encode proteins containing an identical ETS DNA binding domain (Burtis et al., 1990). *E74* mutants display lethality during prepupal and pupal stages, with defects in adult head eversion and leg morphogenesis as well as defects in ecdysteroid-regulated gene expression (Fletcher et al., 1995; Fletcher and Thummel, 1995).

The *E75* ecdysteroid-inducible gene from the 75B early puff encodes three protein isoforms designated E75A, E75B, and E75C (Segraves and Hogness, 1990). These proteins contain the canonical DNA binding domain and ligand binding domain that define members of the nuclear receptor superfamily, although they are referred to as orphan nuclear receptors because a corresponding hormonal ligand has not yet been identified (Mangelsdorf and Evans, 1995). Each E75 isoform is characterized by a unique N-terminal sequence encoded by a distinct 5′ exon (Segraves and Hogness, 1990). These 5′ exons splice to a common set of five 3′ exons for *E75A* and *E75C*, while *E75B* shares only the last four 3′ exons (Figure 1A). As a result of this arrangement, E75B contains only one of the two E75 zinc fingers and is thus incapable of binding DNA. E75B can, however, heterodimerize with the DHR3 ecdysteroid-inducible orphan nuclear receptor and has been detected on salivary gland polytene chromosomes by antibody stains, indicating that it may function at the level of target gene regulation (White et al., 1997).

E75A is transcribed during each stage in the life cycle

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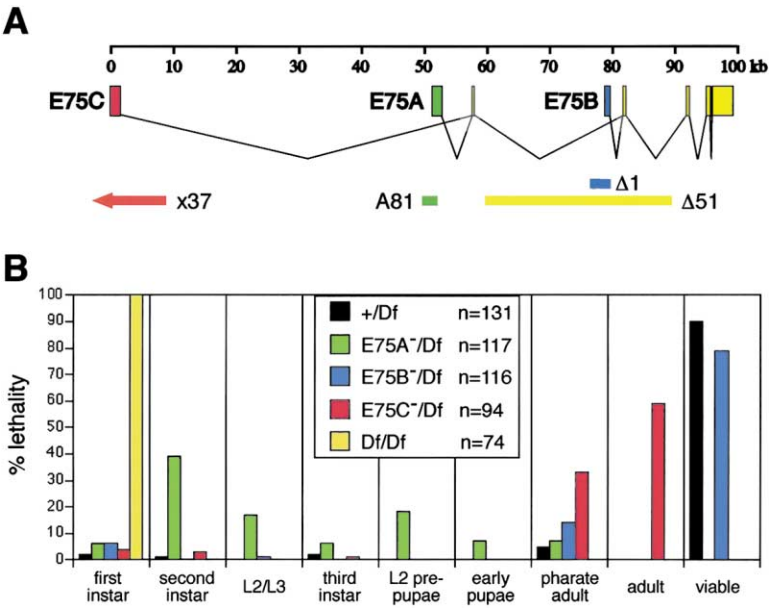

Figure 1. Location of *E75* Isoform-Specific Mutations and Corresponding Lethal Phases

(A) A map of 100 kb of genomic DNA is shown, spanning the *E75* locus. *E75* directs the synthesis of three overlapping transcripts that arise from unique promoters (Segraves and Hogness, 1990). An exon specific for *E75A* (green) and another for *E75C* (red) are each joined to a common set of five 3′ exons (yellow), while the *E75B*-specific exon (blue) is spliced to only the last four 3′ exons. *E75* deletions are depicted as colored bars under the corresponding genomic region. The *E75C* mutation ($E75^{x37}$) is an ~60 kb deletion (red arrow). The *E75A* mutation ($E75^{A81}$) is a 1.8 kb deletion (green bar). The *E75B* mutation ($E75^{\Delta 1}$) is an ~3 kb deletion (blue bar). The common region mutation ($E75^{\Delta 51}$) is an ~30 kb deletion that removes exons shared by all three isoforms (yellow bar). The relative positions of *E75* transcription units are based on the genomic map of Segraves and Hogness (1990), and do not include an ~9 kb roo transposable element that maps ~1.5 kb downstream of the *E75C*-specific region in the reported *Drosophila* genome sequence (Adams et al., 2000).

(B) First instar larvae were selected from crosses that generated the following genotypes: $ry^{506}/E75^{\Delta 51}$ (+/*Df*), $E75^{A81}/E75^{\Delta 51}$ ($E75A^{-}$/*Df*), $E75^{\Delta 1}/E75^{\Delta 51}$ ($E75B^{-}$/*Df*), $E75^{x37}/E75^{\Delta 51}$ ($E75C^{-}$/*Df*), and $E75^{\Delta 51}/E75^{\Delta 51}$ (*Df*/*Df*). Lethality was scored at 24 hr intervals. The stage of development at which an animal died is depicted as a function of the percentage of animals that died at that stage. n refers to the number of animals scored from each genotype. L2/L3 refers to animals that died while molting from the second to third instar. L2 prepupae refers to animals that pupariated from the second instar.

in bursts that accompany ecdysteroid peaks in the life cycle, as well as in late embryos in synchrony with the *E74A* early mRNA (Segraves, 1988; Thummel et al., 1990). The three *E75* transcripts accumulate with distinct kinetics at the onset of metamorphosis, with peaks of *E75A* mRNA in late third instar larvae and late prepupae in synchrony with the ecdysteroid pulses, consistent with its direct induction by ecdysteroids (Andres et al., 1993; Huet et al., 1993; Karim and Thummel, 1992; Segraves and Hogness, 1990). *E75B* mRNA accumulates to peak levels in 2–4 hr prepupae, while *E75C* mRNA peaks in 10–12 hr prepupae.

Relatively little is known about *E75* functions during the *Drosophila* life cycle. Evidence from biochemical and ectopic expression studies indicates that E75B can act as a repressor of the βFTZ-F1 competence factor during metamorphosis (White et al., 1997). Germline clones of *E75* null mutants, missing all three isoforms, lead to arrest during mid-oogenesis, similar to the phenotype of *EcR* mutant germline clones (Buszczak et al., 1999). A zygotic loss of *E75* function results in midgut morphogenesis defects during embryogenesis (Bilder and Scott, 1995). Genetic studies of individual *E75* isoforms, however, and more detailed phenotypic studies at other stages of the life cycle, have not been reported. Here, we describe the phenotypes of null mutations specific to *E75A*, *E75B*, and *E75C*, as well as a mutant in which none of the three E75 isoforms are expressed.

Results

Isolation and Molecular Characterization of *E75* Mutations

Imprecise excision of P elements was used to generate small deletions of sequences specific to either *E75A* or *E75B*, and to create a larger deletion of common region sequences shared by all three E75 isoforms (Figure 1A). $E75^{A81}$ is a 1.8 kb deletion that removes the *E75A* transcription start site along with the 5′-untranslated region and 143 bp of protein-coding sequence (Shilton, 2001). $E75^{\Delta 1}$ is an ~3 kb deletion that removes the *E75B* transcription start site along with most of the first *E75B* exon. $E75^{\Delta 51}$ is an ~30 kb deletion that removes the first exon of *E75B* as well as the adjacent exon, shared by all three *E75* isoforms, that encodes the second zinc finger of the DNA binding domain (Figure 1A). In homozygotes and heteroallelic combinations, $E75^{\Delta 51}$ and $E75^{e213}$, a putative null EMS-induced point mutation in the *E75* common region (Buszczak et al., 1999; Segraves, 1988), lead to similar lethal phases and phenotypes, arguing that the $E75^{\Delta 51}$ mutation inactivates all *E75* functions (data not shown). $E75^{x37}$ is an ~60 kb γ irradiation-induced deletion that removes the *E75C* transcription start site as well as ~10 kb of the downstream primary transcript (Segraves, 1988; W.A.S., unpublished results; Figure 1A). $E75^{x37}$ fails to complement another *E75C* allele, $E75^{e273}$, which is an EMS-induced 63 bp deletion that removes the *E75C* splice donor (Segraves, 1988; data not shown). $E75^{x37}$ and $E75^{e273}$ generate identical lethal phenotypes when maintained over a deletion for the *E75* locus, providing further evidence that *E75C* is the only essential *E75* function affected by the *x37* deficiency.

Similar lethal phases and phenotypes were observed for $E75^{A81}$ and $E75^{x37}$ mutants when examined as either homozygotes or over the $E75^{\Delta 51}$ common region deficiency, suggesting that they are null alleles with respect to the affected isoform. All studies described here use an isoform-specific *E75* mutation over the $E75^{\Delta 51}$ common region deficiency. For simplicity, we refer to $E75^{\Delta 51}$ as

the deficiency (*Df*) for the *E75* locus, $E75^{A81}$/*Df* as *E75A* mutants, $E75^{\Delta 1}$/*Df* as *E75B* mutants, and $E75^{x37}$/*Df* as *E75C* mutants. mRNA corresponding to the affected *E75* isoform was undetectable by Northern blot hybridization in each of these genotypes, consistent with the molecular nature of these lesions and providing further evidence that they are null alleles (data not shown).

E75B Mutants Are Viable while *E75C* Mutants Are Pharate Adult and Adult Lethal

Crosses were set up between control ry^{506}/TM6B *Ubi-GFP*, $E75^{\Delta 1}$/TM6B *Ubi-GFP*, or $E75^{x37}$/TM6B *Ubi-GFP* animals and the deficiency stock $E75^{\Delta 51}$/TM6B *Ubi-GFP* to test for embryonic lethality among the offspring. From the ry^{506} control cross, 26% of the first instar larvae did not express GFP, indicating no significant embryonic lethality. Similar results were obtained from the crosses with *E75B* mutants (23%, n = 90) and *E75C* mutants (25%, n = 78).

E75B and *E75C* mutant first instar larvae were collected at hatching and examined at regular intervals for phenotypes and lethality during later stages of development. *E75B* mutants are viable and fertile, with no detectable phenotypes (Figure 1B). In contrast, *E75C* mutants display lethality during pharate adult and adult stages (Figure 1B). Approximately 33% of *E75C* mutants die as pharate adults, with normal adult pigmentation and fully developed appendages. The remaining *E75C* mutants eclose and are severely uncoordinated, displaying difficulty in walking and an inability to fly. These animals die within a week following eclosion. *E75C* mutant adults appear morphologically normal with the exception of black spots that cover about one quarter of the surface of the eye (data not shown).

E75C Is Required for Maintaining the Transcription of a Subset of Ecdysteroid-Inducible Genes at the Prepupal-Pupal Transition

E75B and *E75C* are both induced by ecdysteroids at the onset of metamorphosis, suggesting that they may function during this stage in development (Huet et al., 1993; Karim and Thummel, 1992). Gain-of-function studies have also implicated *E75B* in contributing to the timing of β*FTZ-F1* expression in mid-prepupae (White et al., 1997). We therefore examined the temporal profiles of ecdysteroid-regulated gene transcription in *E75B* and *E75C* mutant late larvae and prepupae. Total RNA was isolated from control, *E75B*, and *E75C* mutant late third instar larvae staged at −18, −8, and −4 hr relative to puparium formation, as well as from prepupae staged at 2 hr intervals after puparium formation. These RNA samples were analyzed by Northern blot hybridization using radiolabeled probes designed to detect 16 ecdysteroid-regulated transcripts: *EcR*, *BR-C*, *E74A*, *E74B*, *E75A*, *E75B*, *E75C*, *E78B*, *DHR3*, *Imp-E1*, *Fbp-1*, *Sgs-4*, *L71-1*, *L71-3*, β*FTZ-F1*, and *Edg84A* (Andres et al., 1993).

All of the tested transcription units, with the exception of *E75B*, are expressed normally in *E75B* mutant larvae and prepupae (data not shown). We focused our efforts on the temporal profile of β*FTZ-F1* expression in this mutant, preparing several independent Northern blots, one of which utilized prepupae staged at 30 min intervals. No reproducible effects on β*FTZ-F1* expression, however, could be detected (data not shown). We thus conclude that *E75B* is not required for the appropriate timing of β*FTZ-F1* transcription during prepupal development.

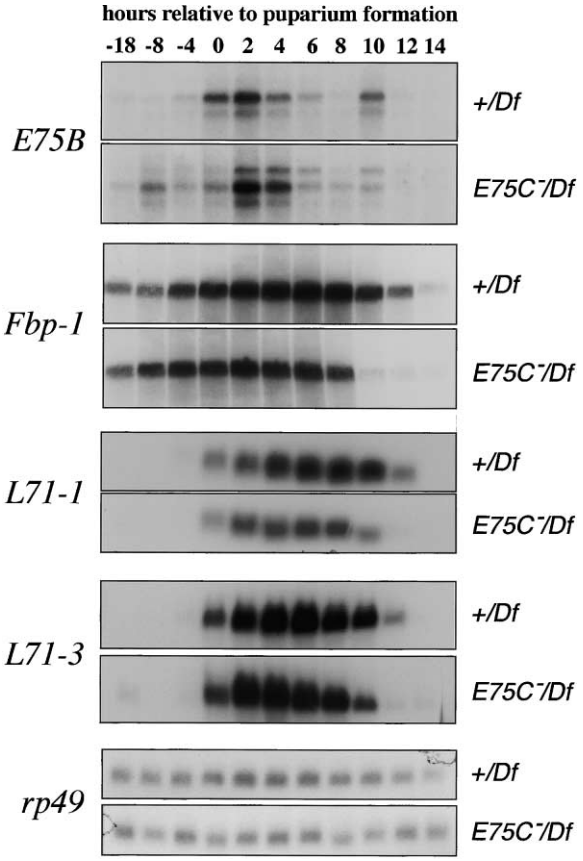

Figure 2. *E75C* Is Required for Sustained Transcription of *E75B*, *Fbp-1*, *L71-1*, and *L71-3* during the Prepupal-Pupal Transition

Developmental times are shown in hours relative to puparium formation. Total RNA was isolated from $ry^{506}/E75^{\Delta 51}$ control animals (+/*Df*) and from $E75^{x37}/E75^{\Delta 51}$ mutant animals ($E75C^{-}$/*Df*), fractionated by formaldehyde gel electrophoresis, and analyzed by Northern blot hybridization. Blots were hybridized with radiolabeled DNA probes to detect the transcription of 16 ecdysteroid-regulated genes. The four affected genes are depicted. *rp49* mRNA is used as a control for loading and transfer.

The tested transcription units are also expressed normally in *E75C* mutants at the onset of metamorphosis, with four exceptions (Figure 2). *E75B* mRNA normally peaks in abundance at 2 hr after puparium formation and is reinduced ~8 hr later, in response to the prepupal ecdysteroid pulse (Huet et al., 1993; Karim and Thummel, 1992; Figure 2). This reinduction is submaximal in *E75C* mutants (Figure 2). Interestingly, the *E75C* mutation has a similar effect on *Fbp-1*, *L71-1*, and *L71-3* transcription at this stage in development (Figure 2). *Fbp-1* encodes a larval serum protein receptor that is induced by ecdysteroids in the fat body of mid-third instar larvae (Burmester et al., 1999), and *L71-1* and *L71-3* are late ecdysteroid-inducible genes that are specifically expressed in prepupal salivary glands (Restifo and Guild, 1986). These genes are normally downregulated in early pupae, ~14 hr after puparium formation.

In *E75C* mutants, however, this repression occurs prematurely, ~10 hr after puparium formation. It is at this time that *E75C* mRNA levels peak in abundance in wild-type animals (Andres et al., 1993; Karim and Thummel, 1992), indicating that *E75C* is normally required to maintain the expression of these genes through the prepupal-pupal transition.

The *E75* Deficiency Causes First Instar Larval Lethality while *E75A* Mutants Die throughout Development

To assess possible embryonic lethality, $E75^{A81}$/TM6B *Ubi-GFP* animals were crossed to the deficiency stock $E75^{\Delta 51}$/TM6B *Ubi-GFP*. From this cross, 20% of the offspring hatched as first instar larvae that did not express GFP (n = 161). Similarly, 20% of the offspring from $E75^{\Delta 51}$/TM6B *Ubi-GFP* adults hatched as first instar larvae that did not express GFP (n = 162). These numbers are lower than the 26% observed in the ry^{506} control cross (n = 150), indicating some embryonic lethality. Embryonic lethality in *E75* common region mutants has been shown to be associated with abnormal midgut morphogenesis (Bilder and Scott, 1995) as well as head involution defects (P. Jenik and W.A.S., unpublished results).

E75A and $E75^{\Delta 51}$ common region mutant first instar larvae were collected at hatching and examined at regular intervals for lethality during later stages of development. $E75^{\Delta 51}$ common region mutants remain as first instar larvae for over a week before dying without any detectable morphological abnormalities (Figure 1B). Some *E75A* mutants also arrest development as first instar larvae, although lethality is also observed at most other stages in the life cycle including second instar larvae, third instar larvae, early pupae, and pharate adults (Figure 1B). Developmental delays, developmental arrests, and molting defects were observed in these animals. Subsets of first and second instar mutant larvae never molt to the next instar, surviving for up to a week before dying. Those that molt do so up to 12 hr late, while some die at the molt (denoted as L2/L3 in Figure 1B). These animals often have malformed mouthhooks or two sets of mouthhooks, indicative of a molting defect. *E75A* mutant pharate adults display no detectable morphological defects, but fail to eclose.

E75A Mutant Second Instar Larvae Can Pupariate without Progressing through the Third Instar

Approximately 20% of *E75A* mutant second instar larvae display a heterochronic phenotype in which they live for several days beyond the time when they should have molted to the third instar, and then pupariate (denoted as L2 prepupae in Figure 1B). These delayed second instar larvae continue to eat and grow, exceeding the size of wild-type second instar larvae, approaching the size of a wild-type late third instar larva. They begin to pupariate ~88 hr after the first-to-second instar larval molt, forming what will be referred to hereafter as L2 prepupae.

We conclude that the L2 prepupae derive from second instar larvae based on three criteria. First, L2 prepupal anterior spiracles do not evert and consist of a single club-shaped spiracular opening, identical to that of second instar larvae (Figures 3B and 3D; Bodenstein, 1965), in contrast to the everted spiracular papillae characteristic of a wild-type prepupa derived from a third instar larva (Figures 3A and 3C). Second, mouthhooks dissected from L2 prepupae, although malformed to varying degrees (Figure 3G), are more similar to wild-type second instar larval mouthhooks, both in size and tooth structure (Figure 3F), than to wild-type third instar larval mouthhooks (Figure 3E). Finally, no ejected mouthhooks or shed cuticle could be found in the media of *E75A* mutant L2 prepupae by the time they pupariated. Remarkably, about 20% of the L2 prepupae (n = 85) develop to the pupal stage as evidenced by head eversion and leg and wing extension, indicating that these animals respond in a relatively normal manner to the prepupal ecdysteroid pulse (Figure 3I).

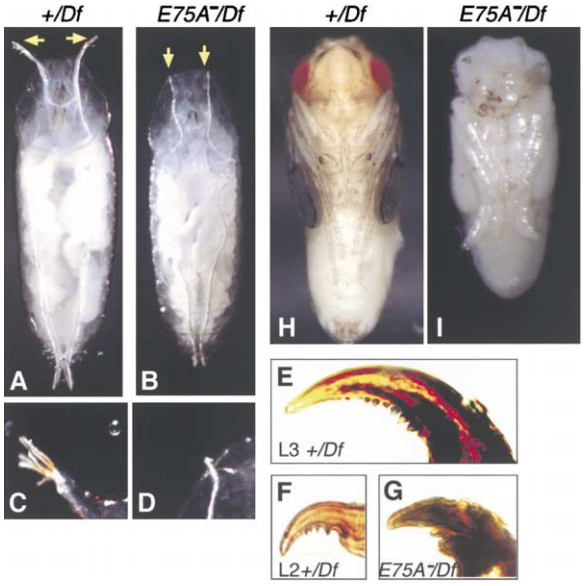

Figure 3. *E75A* Mutant Second Instar Larvae Can Pupariate without Progressing through the Third Instar

A wild-type $ry^{506}/E75^{\Delta 51}$ (+/*Df*) prepupa (A) and $E75^{A81}/E75^{\Delta 51}$ ($E75A^{-}$/*Df*) L2 prepupa (B) are depicted, along with higher magnification images of the anterior spiracles of wild-type (C) and *E75A* mutant (D). Dissected mouthhooks are also depicted from a wild-type third instar larva (E), a wild-type second instar larva (F), and an *E75A* mutant L2 prepupa (G). Some L2 prepupae progress through pupation, as indicated by an everted head and elongated legs and wings (I). A few necrotic patches can be seen in the depicted animal, which was photographed approximately 2 days after pupation. A wild-type late pupa is shown for comparison (H).

E74A and β*FTZ-F1* Are Submaximally Induced in *E75A* Mutant Second Instar Larvae

The majority of *E75A* mutants display defects during the second larval instar (Figure 1B). In an initial effort to understand the molecular basis of these defects, total RNA was isolated from staged control and *E75A* mutant second instar larvae and analyzed by Northern blot hybridization using probes to detect the expression of eight genes: *E74*, β*FTZ-F1*, *EcR*, *usp*, *dare*, *dib*, *Lcp-b*,

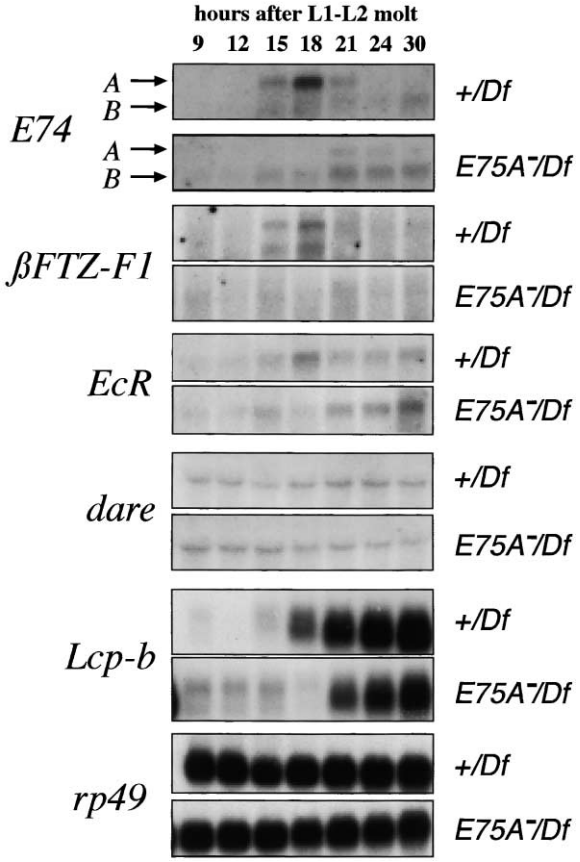

Figure 4. *E75A* Mutant Second Instar Larvae Display Defects in *E74* and β*FTZ-F1* Transcription

Total RNA was isolated from $ry^{506}/E75^{\Delta 51}$control (+/*Df*) and $E75^{A81}/E75^{\Delta 51}$ mutant second instar larvae ($E75A^{-}/Df$), fractionated by formaldehyde gel electrophoresis, and analyzed by Northern blot hybridization. Radiolabeled DNA probes were used to detect *E74*, β*FTZ-F1*, *EcR*, *dare*, and *Lcp-b* mRNA. *rp49* was used as a control for loading and transfer. Developmental times are depicted in hours after the first-to-second instar larval molt.

and *rp49* (Figure 4 and data not shown). We focused on three regulatory genes that are expressed during the second instar: *EcR*, *E74*, and β*FTZ-F1* (Talbot et al., 1993; Thummel et al., 1990; Yamada et al., 2000). These genes are coordinately induced in wild-type second instar larvae 15–18 hr after the molt (Figure 4). Expression of *E74* and β*FTZ-F1* is significantly affected in *E75A* mutant second instar larvae (Figure 4). *E74B* mRNA can be detected throughout the time course, accumulating to higher levels at later times, while *E74A* mRNA accumulation is both significantly reduced and delayed. β*FTZ-F1* mRNA is also significantly reduced in *E75A* mutant second instar larvae (Figure 4). The effects on *EcR* mRNA accumulation, however, are relatively minor, with *EcR* failing to be repressed at later times (Figure 4). These blots were also hybridized to detect *usp* mRNA, which is unaffected by the *E75A* mutation (data not shown). The expression of *EcR* and *usp* mRNA in *E75A* mutant second instar larvae suggests that the defects in this mutant cannot be attributed to a reduced level of ecdysteroid receptor at this stage in development.

The pattern of *E74* transcription seen in *E75A* mutants is similar to that seen in third instar larval organs treated with a low concentration of 20E (Karim and Thummel, 1991). This observation raises the possibility that *E75A* mutant second instar larvae might be ecdysteroid deficient. This proposal is further supported by the lethal phenotypes of *E75A* mutants which resemble those seen in ecdysteroid-deficient mutants (Freeman et al., 1999; Sliter and Gilbert, 1992; Venkatesh and Hasan, 1997). Accordingly, we examined the expression of two genes that are known to be directly involved in the ecdysteroidogenic pathway in *Drosophila*: *dare* and *disembodied* (*dib*). *dare* encodes the *Drosophila* ortholog of adrenodoxin reductase, a mammalian enzyme that plays a central role in vertebrate steroid hormone biosynthesis by transferring electrons to all known mitochondrial cytochrome P450s (Freeman et al., 1999). Genetic studies of *dare* mutants suggest that this gene plays a similar role in *Drosophila* ecdysteroid biosynthesis. *dib* encodes a presumptive target for *dare* action, a cytochrome P450 that is essential for ecdysteroid biosynthesis in *Drosophila* (Chavez et al., 2000). Both *dare* and *dib* mRNA, however, are expressed throughout the second larval instar and appear to be unaffected by the *E75A* mutation (Figure 4 and data not shown). Similar results were obtained with separate collections of animals and with poly(A)$^{+}$ RNA from *E75A* mutant second instar larvae (data not shown).

Lcp-b expression was also examined in *E75A* mutant second instar larvae. This gene encodes a larval cuticle protein that is induced during the latter half of the second instar (Charles et al., 1998). *Lcp-b* mRNA is upregulated at 18 hr after the molt, in synchrony with *EcR*, *E74A*, and β*FTZ-F1*, and accumulates to higher levels throughout the second instar, consistent with earlier results (Figure 4; Charles et al., 1998). *Lcp-b* transcription is slightly delayed and reduced in *E75A* mutant larvae but otherwise expressed normally, indicating that these animals can faithfully express a marker for the second instar stage (Figure 4).

E75A Mutant Second Instar Larvae Express a Third Instar Genetic Program

A subset of *E75A* mutant second instar larvae fails to molt to the third instar and pupariate, forming L2 prepupae (Figure 1B). To determine whether these animals execute genetic programs specific to later stages of development, we examined the patterns of *E74*, *Sgs-4*, and *Fbp-1* transcription in *E75A* mutant second instar larvae. These ecdysteroid-inducible genes are normally expressed during the second half of third larval instar and thus provide molecular markers for animals that are progressing toward the onset of metamorphosis (Andres et al., 1993).

Total RNA was collected from control third instar larvae staged from 24 to 44 hr after the second-to-third instar molt, and *E75A* mutant second instar larvae staged from 48 to 88 hr after the first-to-second instar molt. Control larvae begin to pupariate at ~48 hr after the second-to-third instar molt, while the majority of *E75A* mutant second instar larvae begin to pupariate after 88 hr. RNA extracted from both sets of animals was analyzed by Northern blot hybridization (Figure 5).

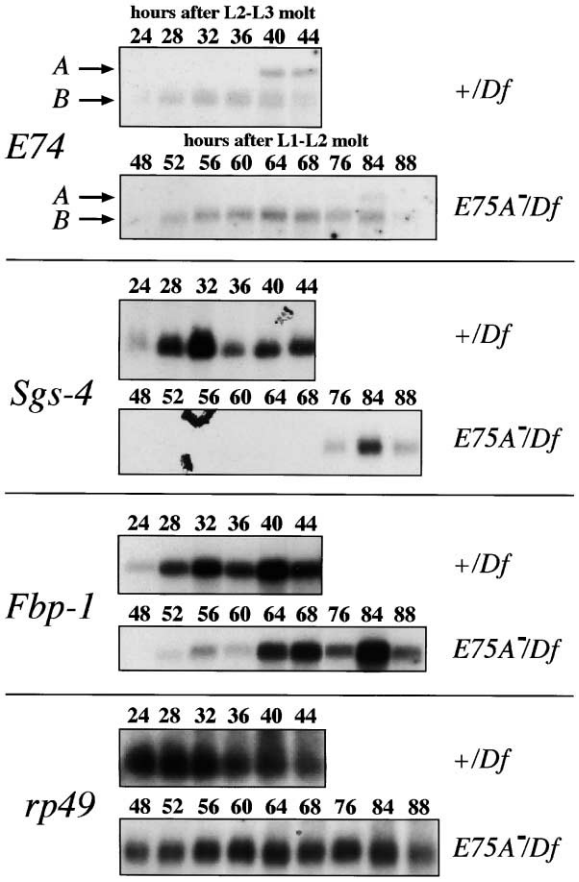

Figure 5. Delayed *E75A* Mutant Second Instar Larvae Express Genes Specific to the Third Instar

Total RNA was isolated from $ry^{506}/E75^{\Delta 51}$ control third instar larvae (+/*Df*) and from $E75^{A81}/E75^{\Delta 51}$ mutant second instar larvae (*E75A*$^{-}$/*Df*), fractionated by formaldehyde gel electrophoresis, and analyzed by Northern blot hybridization. Radiolabeled DNA probes were used to detect *E74A*, *E74B*, *Sgs-4*, and *Fbp-1* mRNA. *rp49* was used as a control for loading and transfer. Control larvae are staged in hours after the second-to-third instar larval molt and *E75A* mutant larvae are staged in hours after the first-to-second instar larval molt. The RNA level in the 88 hr time point in *E75A* mutant larvae is low relative to the other samples. The variable levels of *Sgs-4* and *Fbp-1* mRNA detected at different time points in *E75A* mutants is most likely due to developmental asynchrony in each collection of animals.

E74B is expressed throughout the second half of the third larval instar in wild-type animals, and begins to be repressed as *E74A* mRNA is induced by the high-titer late larval ecdysteroid pulse (Figure 5). A similar pattern is seen in *E75A* mutant second instar larvae, although *E74A* induction is significantly delayed and reduced, detectable at 84 hr after the first-to-second instar molt (Figure 5). Both *Sgs-4* and *Fbp-1* are also induced in *E75A* mutant second instar larvae, although their normally coordinate induction is disrupted, with *Fbp-1* induced 56–64 hr after the molt, and *Sgs-4* induced 76–84 hr after the molt in mutant animals (Figure 5). The expression of *Sgs-4* and *Fbp-1* in *E75A* mutant second instar larvae indicates that they are capable of inducing genetic programs specific to the third instar stage.

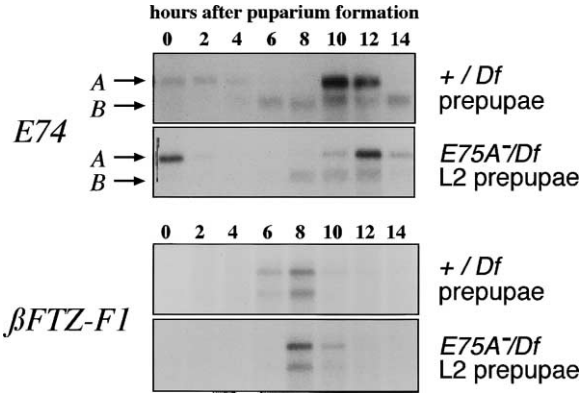

Figure 6. *E75A* Mutant L2 Prepupae Have Normal Temporal Profiles of *E74* and β*FTZ-F1* Transcription

Total RNA was isolated from $ry^{506}E75^{\Delta 51}$ (+/*Df*) control prepupae and $E75^{A81}/E75^{\Delta 51}$ L2 prepupae (*E75A*$^{-}$/*Df*), fractionated by formaldehyde gel electrophoresis, and analyzed by Northern blot hybridization. Radiolabeled DNA probes were used to detect *E74A*, *E74B*, and β*FTZ-F1* transcription. *rp49* was used as a control for loading and transfer. Times are depicted in hours after puparium formation.

E75A Mutant L2 Prepupae Display Normal Prepupal Temporal Patterns of *E74* and β*FTZ-F1* Transcription

Approximately 20% of *E75A* mutant L2 prepupae undergo head eversion, forming pupae with elongated legs and wings (Figure 3I). In an effort to determine whether these developmental changes reflect normal transcriptional responses to ecdysteroids, we examined the expression of two key ecdysteroid-regulated genes that respond to dynamic changes in ecdysteroid titer in prepupae: *E74* and β*FTZ-F1*. *E74A* is expressed in newly formed prepupae and is repressed as *E74B* is induced by the rising ecdysteroid titer during prepupal development. *E74B* is then repressed as *E74A* is induced by the prepupal ecdysteroid pulse, followed by rapid repression of *E74A* and reinduction of *E74B* (Karim and Thummel, 1991). In contrast, β*FTZ-F1* expression is restricted to the interval of low-ecdysteroid titer in mid-prepupae (Woodard et al., 1994).

Total RNA was isolated from control prepupae and pupae staged at 2 hr intervals from 0 to 14 hr after puparium formation, as well as from staged *E75A* mutant L2 prepupae, and analyzed by Northern blot hybridization to detect *E74* and β*FTZ-F1* transcription (Figure 6). Remarkably, the L2 prepupae display relatively normal patterns of *E74* and β*FTZ-F1* expression, with a delay of ~2 hr in *E74A* mRNA accumulation (Figure 6). This observation suggests that the *E75A* mutants that pupariate from the second instar can execute appropriate changes in ecdysteroid titer during the onset of metamorphosis.

Molting Defects in *E75A* Mutants Can Be Rescued by Feeding Ecdysteroids

The developmental delays, developmental arrests, and molting defects seen in *E75A* mutants are characteristic of an ecdysteroid deficiency (Freeman et al., 1999; Sliter and Gilbert, 1992; Venkatesh and Hasan, 1997). This

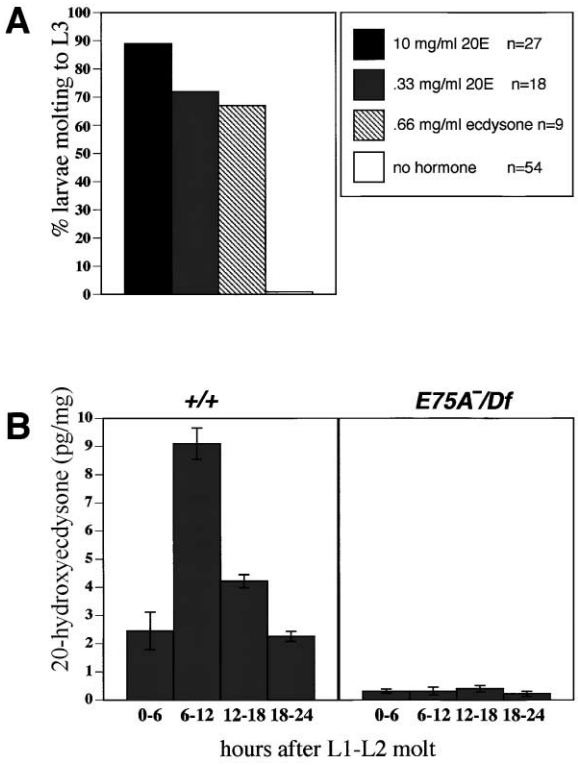

Figure 7. *E75A* Mutant Second Instar Larvae Are Ecdysteroid Deficient

(A) Molting defects in *E75A* mutant second instar larvae are rescued by feeding ecdysteroids. *E75A* mutant second instar larvae ($E75^{A81}$/$E75^{\Delta 51}$) were collected at 66 hr after egg laying and transferred to yeast paste supplemented with either 3.3% ethanol (control), 10 mg/ml 20E, 0.33 mg/ml 20E, or 0.66 mg/ml ecdysone. Animals were transferred to fresh unsupplemented yeast paste 6 hr later and scored for molting to the third instar (L3) after approximately 18 hr. (B) *E75A* mutant second instar larvae have a reduced ecdysteroid titer. w^{1118} control (+/+) and $E75^{A81}/E75^{\Delta 51}$ ($E75A^{-}/Df$) larvae were collected at 6 hr intervals after the first-to-second instar molt. Ecdysteroid titers in organic extracts from these animals were determined by enzyme immunoassay using a monoclonal antibody directed against 20E. The results are depicted as pg of 20-hydroxyecdysone/mg initial body weight on the *y* axis and hours after the molt on the *x* axis.

conclusion is further supported by the pattern of *E74* transcription in *E75A* mutant second instar larvae (Figure 4; Karim and Thummel, 1991). In order to test this possibility, we attempted to rescue the second-to-third instar larval molt by feeding ecdysteroids to *E75A* mutant second instar larvae. Second instar larvae staged 12–18 hr after the molt were transferred to yeast paste supplemented with either no hormone, 0.33 mg/ml 20E, 10 mg/ml 20E, or 0.66 mg/ml ecdysone. The animals were transferred to regular yeast paste after 6 hr in order to simulate the hormone pulse that triggers the molt, and then scored for animals that molted to the third instar after 18 hr. Almost all *E75A* mutant second instar larvae that were maintained on food without ecdysteroids failed to molt, either staying as second instar larvae or forming L2 prepupae (Figure 7A). In contrast, the majority of larvae fed either ecdysone or 20E molted properly (Figure 7A). Interestingly, about half of the mutant animals that molted to the third instar continued to develop to pupal and pharate adult stages, consistent with a critical role for *E75A* during the second larval instar.

E75A Mutant Second Instar Larvae Have Reduced Ecdysteroid Titers

Our studies of *E75A* mutant second instar larvae suggest that they could have a reduced ecdysteroid titer at this stage of development. As a direct test of this hypothesis, we measured the ecdysteroid titer in these animals using an enzyme immunoassay (EIA). Control and *E75A* mutant second instar larvae were collected at 0–6, 6–12, 12–18, and 18–24 hr after the first-to-second instar larval molt. Organic extracts were prepared from these animals and the ecdysteroid titer was measured by EIA using a monoclonal antibody directed against 20E. Wild-type larvae show a peak of 20E at 6–12 hr after the molt, with the titer decreasing toward the end of the instar, as observed in an earlier study (Kraminsky et al., 1980; Figure 7B). In contrast, this peak is eliminated in *E75A* mutants, which also show a reduced basal level of 20E at all stages (Figure 7B). To confirm these results, the EIA was repeated using a second set of control and *E75A* mutant larvae, collected at 0–12, 12–24, and 24–36 hr after the first-to-second instar larval molt. Similar results were obtained from these animals, confirming the data shown in Figure 7B and indicating that the ecdysteroid peak in *E75A* mutants is not delayed until 24–36 hr after the molt (data not shown).

Discussion

Extensive studies have focused on the 74EF and 75B early ecdysteroid-inducible puffs in the salivary gland polytene chromosomes, providing insights into the molecular mechanisms of ecdysteroid action in insects as well as steroid hormone signaling in vertebrates (Ashburner et al., 1974; Yamamoto and Alberts, 1976). Although functions for the *E74* early gene from the 74EF puff have been described (Fletcher et al., 1995), relatively little is known about the roles of the E75 orphan nuclear receptors during development. We show here that *E75B* mutants are viable and fertile, suggesting that this gene functions in a redundant pathway during development, while *E75C* mutants die as adults. In contrast, most *E75A* mutants die as delayed second instar larvae with a reduced ecdysteroid titer, or arrest during the molt to the third instar. Remarkably, some *E75A* mutant second instar larvae express genes characteristic of the third instar and pupariate without progressing through a molt, indicating that molting can be uncoupled from the onset of metamorphosis. This study provides a new direction for understanding the functions of early ecdysteroid-inducible regulatory genes, positioning the E75A orphan nuclear receptor upstream from the signal that induces its expression, defining its action in a feed-forward pathway to amplify or maintain ecdysteroid titers during *Drosophila* larval development.

E75B Exerts No Essential Functions during the Fly Life Cycle

E75B null mutants show no detectable phenotypes. In addition, the expression of 16 ecdysteroid-regulated genes at the onset of metamorphosis is normal in these mutants (data not shown). Interestingly, β*FTZ-F1* is among the genes that remain unaffected by the *E75B* mutation. Ectopic expression and biochemical studies have shown that E75B can directly interact with the DHR3 orphan nuclear receptor and thereby block its ability to induce β*FTZ-F1* in mid-prepupae (White et al., 1997). These observations led to the proposal that the timing of β*FTZ-F1* expression is dependent on appropriate decay of the E75B repressor. We, however, find no reproducible temporal shift in β*FTZ-F1* expression in *E75B* mutant prepupae, indicating that, while *E75B* may be sufficient to repress β*FTZ-F1*, it is not necessary for this response. This conclusion, combined with the absence of obvious phenotypic effects caused by the *E75B* mutation, raises the possibility that *E75B* acts in a functionally redundant pathway. *E78B* is an ideal candidate for exerting this redundant activity. Like *E75B*, *E78B* encodes a homolog of the vertebrate Rev-Erb orphan nuclear receptor (NR1D1), and is a truncated isoform that lacks a DNA binding domain (Stone and Thummel, 1993). *E78B* is expressed in synchrony with *E75B* in early prepupae (Karim and Thummel, 1992; Stone and Thummel, 1993). In addition, *E78B* null mutants display no detectable phenotypes, like *E75B* mutants, suggesting that its function is complemented by another factor (Russell et al., 1996). An effort is currently underway to inactivate both *E75B* and *E78B* in order to determine whether they act in a redundant manner during development (G. Lam and C.S.T., unpublished results).

E75C Is Required for Pharate Adult and Adult Viability

E75C mutants die as pharate adults or within a few days following eclosion (Figure 1B). These animals are morphologically normal except for black spots that cover up to one quarter of the eye, but are weak, unable to fly, and severely uncoordinated. Earlier development in these mutants appears to proceed normally. Consistent with this observation, most of the 16 ecdysteroid-regulated transcripts examined in *E75C* mutant late third instar larvae and prepupae displayed normal temporal patterns of expression (Figure 2). The expression of four transcripts, however, fails to be maintained through the prepupal-pupal transition in *E75C* mutants: *E75B*, *Fbp-1*, *L71-1*, and *L71-3*. This coordinate misregulation suggests that the brief peak of ecdysteroid-induced *E75C* expression in ~10 hr prepupae is required for the continued expression of a subset of ecdysteroid target genes. It is unclear, however, whether these relatively subtle effects on gene expression might be causally related to the late developmental defects observed in *E75C* mutants.

Similarities between the *E75C* adult phenotype and the phenotype exhibited by hypomorphic *dare* alleles raises the possibility that the adult lethality of *E75C* mutants may result from an ecdysteroid deficiency. *dare* mutant adults are unable to walk or fly, exhibiting twitching of the legs and wings, and die within a week following eclosion (Freeman et al., 1999). These *dare* mutant phenotypes appear to arise from progressive degeneration of the CNS, starting at adult eclosion. Future studies could provide a basis for determining whether *E75C* and *dare* might function together to control ecdysteroid titers during pupal and adult development.

E75A Mutant Larvae Are Ecdysteroid Deficient

Most *E75A* mutants display defects during the second larval instar, either failing to molt to the third instar, arresting at the molt, or forming prepupae directly from the second instar (Figure 1B). *E75A* mutant larvae develop asynchronously and can molt up to 1 day late. Larvae that do not molt can live for up to a week before dying. These phenotypes resemble those seen with a temperature-sensitive *dre-4* mutant as well as hypomorphic alleles of *itpr* and null alleles of *dare*. The *dre4* gene has not yet been isolated, although its function is required for ecdysteroid pulses throughout the life cycle (Sliter and Gilbert, 1992). *itpr* encodes an intracellular calcium channel, the inositol 1,4,5-triphosphate (IP_3) receptor, that is expressed in the ring gland and appears to be required for ecdysteroid biosynthesis (Venkatesh and Hasan, 1997). Consistent with their proposed functions, the molting defects in *itpr* and *dare* mutant larvae can be rescued by providing ecdysteroids in the culture medium (Freeman et al., 1999; Venkatesh and Hasan, 1997). Similarly, we have shown that *E75A* mutant second instar larvae can molt to the third instar when fed ecdysteroids (Figure 7A), suggesting that an ecdysteroid deficiency is the primary cause of the observed developmental defects at this stage. More direct evidence for this conclusion is provided by an enzyme immunoassay which indicates that the ecdysteroid pulse is essentially eliminated in *E75A* mutant second instar larvae (Figure 7B). The ring gland, as well as other larval tissues, appears normal in *E75A* mutants, indicating that, like other early genes, *E75A* does not play a role in their growth or development (data not shown). Rather, we conclude that *E75A* is required for appropriate ecdysteroid biosynthesis or release during larval development.

While our studies define an essential role for *E75A* in directing ecdysteroid pulses during larval development, they do not preclude additional functions for this gene during the life cycle—functions that could be masked by the early lethality of *E75A* mutants. Like other early genes, *E75A* is expressed widely in third instar larvae, in contrast to *itpr* and *dare* which are expressed primarily in the ring gland (Freeman et al., 1999; Venkatesh and Hasan, 1997). *E75A* transcripts have been detected in the salivary glands, gut, Malpighian tubules, fat bodies, and imaginal discs (Huet et al., 1993; Segraves, 1988), reflecting the widespread expression of E75A protein detected by antibody stains, including abundant expression in the ring gland (T. Watanabe, personal communication). E75A protein is also bound to multiple sites in the larval salivary gland polytene chromosomes (Hill et al., 1993). These observations suggest that *E75A* may play additional roles beyond those revealed by this study, possibly in a redundant manner with other E75 isoforms or in combination with other early ecdysteroid-inducible genes.

E75A and *E75C* May Exert Redundant Functions during the Onset of Metamorphosis

The *E75* mutant that is missing all three E75 isoforms dies after a prolonged first instar, failing to molt to the second instar, similar to *dre4* and *itpr* null mutants (Sliter and Gilbert, 1992; Venkatesh and Hasan, 1997; Figure 1B). In addition, the earliest phenotypes observed in *E75* common region mutants—embryonic lethality with head involution and midgut morphogenesis defects—resemble the lethal phenotypes of *dib* mutant embryos, mutants that are missing a key cytochrome P450 required for ecdysteroid biosynthesis (Chavez et al., 2000). These observations indicate that the *E75* locus may play an essential role in maintaining ecdysteroid titers through embryonic and larval development. The individual E75 isoforms, however, appear to contribute to this regulatory function in a redundant manner because the highly penetrant early lethality associated with the *E75* common region mutation is not seen with mutations in any of the individual *E75* isoforms (Figure 1B). We conclude that *E75A* and *E75C* are good candidates for exerting this redundant activity by virtue of their identical DNA binding domain.

Redundant interactions between *E75A* and *E75C* may explain the apparent stage specificity of the *E75A* mutant phenotypes. *E75A* and *E75C* mutants display no defects at puparium formation or head eversion, key developmental transitions triggered by ecdysteroid pulses at the onset of metamorphosis. Indeed, most *E75A* mutants progress through to pupal stages once they have passed beyond the second instar (Figure 1B). Similarly, exposure of *E75A* mutant second instar larvae to a single 6 hr treatment with ecdysteroids is sufficient to rescue many animals through to pupal and pharate adult stages (Figure 7A). In addition, *E75A* mutant L2 prepupae appear to execute relatively normal changes in ecdysteroid titer at the onset of metamorphosis, as indicated by the patterns of *E74* and β*FTZ-F1* transcription (Figure 6). We thus propose that *E75A* and *E75C* act in a redundant manner to maintain ecdysteroid titers during the onset of metamorphosis, explaining why mutations in either function survive these stages in the life cycle.

E75A Mutants Uncouple Molting from Entry into Metamorphosis

E75A mutant second instar larvae that fail to molt continue to grow, approaching the size of a wild-type late third instar larva. Remarkably, these delayed second instar larvae express markers that are specific to the latter half of the third instar—the fat body-specific *Fbp-1* larval serum protein receptor gene and the *Sgs-4* salivary gland glue protein gene (Figure 5). These changes in gene expression, in apparent preparation for metamorphosis, are consistent with the ability of these second instar larvae to pupariate and progress through head eversion, dying as early pupae (Figures 1B and 3). *E75A* mutant L2 prepupae display normal temporal patterns of *E74* and β*FTZ-F1* transcription through prepupal and early pupal stages, with an ~2 hr delay relative to control animals (Figure 6). These observations suggest that these mutants can reset their endocrinological clock to execute the proper ecdysteroid pulses that trigger puparium formation and pupation.

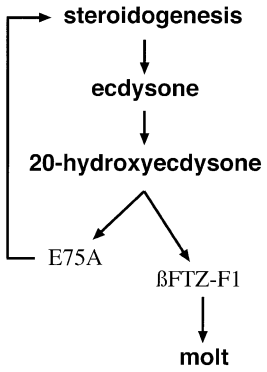

Figure 8. A Feed-Forward Model for *E75A* Function in Steroidogenesis

We propose that *E75A* acts in a feed-forward pathway to amplify or maintain the initial ecdysteroid signal. It could exert this function by directly inducing genes that encode steroidogenic enzymes within the ring gland. These enzymes would then lead to an increase in the ecdysone precursor that is subsequently converted to 20E, the active form of the hormone. The molting defects seen in *E75A* mutants are due to submaximal ecdysteroid induction of β*FTZ-F1* expression.

The ability of *E75A* mutant animals to pupariate directly from the second instar indicates that molting can be uncoupled from progression through the onset of metamorphosis. This appears to be a manifestation of the reduced ecdysteroid titer in these mutants, as pupariating second instar larvae have also been reported in *dre4* and *itpr* mutants, although they were not characterized in any detail (Sliter and Gilbert, 1992; Venkatesh and Hasan, 1997). The reduced ecdysteroid titer in *E75A* mutant second instar larvae apparently causes these animals to miss this cue that would normally trigger the molt to the third instar. In spite of this, however, these animals can still acquire third instar identity and progress relatively normally, albeit with a significant developmental delay that is evident both in terms of *E74A* induction in staged larvae (Figure 5), as well as the time to puparium formation, which is at least 16 hr later than puparium formation in wild-type animals. This conclusion suggests that larval molting is an epidermal response that has few, if any, consequences for temporal progression throughout the rest of the organism. Moreover, the observation that a reduced ecdysteroid titer can lead to a heterochronic phenotype at the onset of metamorphosis defines a critical role for ecdysteroid pulses in defining not just timing but also the character of the major developmental transitions in the fly life cycle (Thummel, 2001).

E75A Acts in a Feed-Forward Pathway for Ecdysteroid Biosynthesis

The observation that *E75A* expression is induced directly by ecdysteroids (Segraves and Hogness, 1990), combined with its requirement for appropriate ecdysteroid titers during larval development, leads us to propose that *E75A* functions in a feed-forward pathway to maintain or amplify ecdysteroid pulses during *Drosophila* larval development (Figure 8). The most direct means by which *E75A* could exert this regulatory function would

be through transcriptional control of genes that encode steroidogenic enzymes (Figure 8). We examined the transcription of *dare* and *dib* in *E75A* mutant second instar larvae as a means of testing this hypothesis, but detected no effects on their expression (Figure 4 and data not shown). Although *dare* and *dib* are the only members of the ecdysteroid biosynthetic pathway that have been characterized at the molecular level, there are other genes in this pathway that could be dependent on *E75A* function. These include members of the so-called "Halloween" class of genes, such as *spook*, *shroud*, and *phantom*, which display lethal mutant phenotypes similar to those of *dib* (Chavez et al., 2000).

The reduced level of β*FTZ-F1* expression in *E75A* mutant second instar larvae provides a functional link to explain the molting defects in these animals (Figure 4). A study by Yamada et al. (2000) has shown that β*FTZ-F1* is required for larval molting. Moreover, β*FTZ-F1* can directly regulate the *Edg84A* pupal cuticle gene, and ectopic overexpression of β*FTZ-F1* leads to an abnormal larval cuticle structure (Murata et al., 1996; Yamada et al., 2000). Taken together, these observations suggest that β*FTZ-F1* plays a key role in controlling larval molts, directly regulating larval cuticle gene expression. The severe reduction in β*FTZ-F1* expression in *E75A* mutant larvae is thus consistent with the inability of these animals to molt. We also conclude that *E75A* does not directly regulate β*FTZ-F1*, since molting can be rescued by feeding ecdysteroids to *E75A* mutant larvae (Figure 7A). This experiment places β*FTZ-F1* downstream from ecdysteroid signaling and *E75A* expression, indirectly dependent on *E75A* activity (Figure 8).

Our studies of *E75* provide a new direction for understanding early gene function within the ecdysteroid-triggered regulatory cascades. Previous work has indicated that early ecdysteroid-inducible genes operate downstream from the ecdysteroid receptor, coordinating the expression of secondary response late genes that, in turn, execute the appropriate biological responses to each hormone pulse during development (Russell and Ashburner, 1996; Thummel, 1996). Our characterization of *E75* functions indicates that early genes not only transduce the ecdysteroid signal but can also affect the signal itself, through feedback regulation. Further studies of *E75* should provide a molecular framework for understanding the genetic control of steroidogenesis in insects. In addition, the heterochronic phenotypes in *E75A* mutants provide a basis for understanding how key developmental landmarks, such as molting, are linked to temporal progression through the insect life cycle.

Experimental Procedures

Drosophila Stocks

Wild-type controls were either ry^{506} or w^{1118}. $E75^{A81}$ was created by imprecise excision of the *l(3)0225* ry^{+} P element that maps 800 bp upstream from the start site of *E75A* transcription. Southern blot hybridizations and DNA sequence analysis indicate that $E75^{A81}$ is a 1792 bp deletion that extends from ~1.3 kb upstream of the *E75A* transcription start site to 143 bp downstream from the start codon. $E75^{\Delta 51}$ was created by imprecise excision of the *l(3)3338* ry^{+} P element that maps ~3.5 kb downstream of the second *E75A* exon (W.A.S. et al., unpublished results). This exon, which is shared between *E75A* and *E75C*, encodes the first zinc finger of the E75 DNA binding domain. Southern blot analysis demonstrated that the $E75^{\Delta 51}$ deletion removes ~30 kb of DNA as shown in Figure 1A. $E75^{\Delta 1}$ was created by imprecise excision of the *l(3)7041* ry^{+} P element that maps immediately upstream of the *E75B* start site, and is an ~3 kb deletion that removes the *E75B* promoter and most of the *E75B* 5′ exon. $E75^{x37}$ was isolated by γ-ray mutagenesis of *st in ri* p^{p} sbd^{2} males and screening for lethality over a *ru h* W^{R10} sbd^{2} Tu^{2} chromosome (Segraves, 1988). Southern blot analysis revealed that $E75^{x37}$ is an ~60 kb deletion that maps to the region shown in Figure 1A (Segraves, 1988).

Lethal Phase Analysis

E75 mutations were maintained over the third chromosome balancer, TM6B *P{*w^{+}*-Ubi-GFP.S65T},* Tb^{1} (stock 4887, Bloomington stock collection), which allows mutant larvae to be distinguished from their balanced siblings by the lack of GFP expression. Flies carrying the $E75^{\Delta 51}$ allele were crossed to each of the *E75* isoform-specific mutants. To assess embryonic lethality, embryos were collected from these crosses at 6 hr intervals and allowed to develop for 24 hr at 25°C, after which mutant first instar larvae were counted. To assess lethality at later stages of development, mutant first instar larvae were isolated and maintained on yeast paste. Living animals were transferred to fresh yeast paste every 24 hr, while dead animals were scored for the stage of lethality and visible phenotypes. It should be noted that the lethal phases of $E75^{A81}$ as well as $E75^{\Delta 51}$ and other strong common region mutants were consistently earlier in studies conducted at Yale University, with putative *E75* common region null mutants showing embryonic lethality and $E75^{A81}$ mutants showing earlier larval lethality (P. Jenik and W.A.S., unpublished results). Under growth conditions at the University of Utah, however, $E75^{\Delta 51}$ and $E75^{A81}$ mutants displayed the lower levels of embryonic and larval lethality reported in this manuscript. It thus appears that environmental parameters can affect the severity of the *E75* mutant phenotypes. Similar results have been seen with mutations in *DHR78* and *rigor mortis*, two genes that, like *E75*, function in ecdysteroid signaling pathways (A. Andres, J. Gates, and C.S.T., unpublished results).

Staging of *E75* Mutant Larvae and Prepupae

First or second instar larvae were collected on yeast paste on a moist sheet of black Whatman filter paper. To maintain proper humidity, the filter paper was placed inside a 50 ml glass beaker in a humidified chamber maintained at 25°C. Larvae were checked at 3 hr intervals for molting, and newly molted larvae were transferred to fresh yeast paste and allowed to develop for the desired time relative to the molt. Late third instar larvae were staged as described (Andres and Thummel, 1994).

Northern Blot Hybridizations

Total RNA was extracted from staged larvae and prepupae as described (Andres and Thummel, 1994). Fifteen micrograms of each RNA sample was fractionated by formaldehyde agarose gel electrophoresis, transferred to a nylon membrane, and UV crosslinked using a Stratalinker on autocrosslink. Northern blots were sequentially stripped and hybridized with radioactive probes that were prepared as described (Andres et al., 1993).

Ecdysteroid Feeding Experiments

E75A mutant larvae were collected at 18 hr after the first-to-second instar molt, and divided into two groups. One group of animals was fed yeast paste with a final concentration of 3.3% ethanol (50 mg dry yeast, 95 μl water, and 5 μl 100% ethanol), while the other group of animals was fed yeast paste with a final concentration of 0.33 mg/ml 20-hydroxyecdysone in 3.3% ethanol (50 mg dry yeast, 95 μl water, and 5 μl of 10 mg/ml 20-hydroxyecdysone [Sigma] in 100% ethanol) or 10 mg/ml 20-hydroxyecdysone or 0.66 mg/ml ecdysone (Fluka). Larvae were transferred to fresh yeast paste without ecdysteroids after 6 hr, in order to simulate a high-titer hormone pulse. These larvae were scored 18 hr later for those animals that successfully molted to the third instar.

Ecdysteroid Titer Measurements

Control (w^{1118}) and *E75A* mutant ($E75^{A81}/E75^{\Delta 51}$) second instar larvae were collected at 0–6, 6–12, 12–18, and 18–24 hr after the first-to-second instar larval molt and stored in Eppendorf microcentrifuge tubes that were weighed before and after the addition of larvae in order to determine the weight of the animals. Samples were frozen at −80° and then homogenized in 0.6 ml methanol in a 2 ml glass dounce using a B pestle and incubated at room temperature for 4 hr under constant agitation. This suspension was centrifuged at 14,000 rpm for 30 min, after which the supernatant was saved and the pellet was incubated overnight with another 0.6 ml methanol. Both supernatants were combined and dried in a speed vac. Ecdysteroid titers were determined by an enzyme immunoassay (Aribi et al., 1997; Pascual et al., 1995), using 20-hydroxyecdysone-peroxidase conjugate as a tracer (a gift from J.-P. Delbecque) and EC19 monoclonal antibody directed against 20E (a gift from J.-P. Delbecque). Samples were solubilized in a phosphate buffer to a final concentration of 25 mg initial body weight/ml and added to microtiter plates that had been coated with secondary anti-IgG antibodies. A known quantity of tracer and EC19 antibody were added and the plates were incubated at room temperature for 3 hr. The plates were then washed several times and bound peroxidase activity was detected using tetramethylbenzidine (Sigma) as a substrate. A microplate reader was used to analyze the data and results were compared with readings from standardized concentrations of 20E (Sigma).

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